

Specific Induction of Intracellular Calcium Oscillations by Complement Membrane Attack on Oligodendroglia

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Oligodendroglia (ODG) are unique among glial cell types in their capacity to activate complement in the absence of antibody, causing insertion of the potentially damaging membrane attack complex (MAC) into the plasma membrane. Using microfluorimetry of indo-1 fluorescence we have detected a complex oscillatory $[Ca^{2+}]_i$ response in ODG following exposure to sublethal dilutions of serum-derived complement. Oscillations were transitory and preceded complete and stable return to resting $[Ca^{2+}]_i$ levels, whereas nonoscillating ODG underwent rapid lysis. Depletion of the terminal complement component C9 from serum removed the oscillatory stimulus, which could be restored by reconstitution with purified C9. Exposure to the C9-homologous peptide melittin produced $[Ca^{2+}]_i$ oscillations similar in pattern to those induced by whole serum. However, this type of response could not be reproduced by Ca^{2+} ionophores or mechanical wounding, suggesting that oscillations cannot be provoked by Ca^{2+} influx alone and depend on the presence of the MAC or a pore-forming lesion. Oscillations were not prevented in the continuous presence of caffeine, demonstrating independence from caffeine-releasable intracellular stores. Inhibition of the endoplasmic reticular Ca^{2+} -ATPase with thapsigargin produced an abrupt elevation in $[Ca^{2+}]_i$, but did not alter the latency between exposure to serum and the initial complement-induced transient. However, the slope of this initial transient was considerably reduced and oscillations suppressed, demonstrating dependence of the oscillatory mechanism on functional endoplasmic reticular Ca^{2+} stores. The coincidence of ODG recovery with oscillating $[Ca^{2+}]_i$ suggests that the complex calcium signal that follows MAC attack may stimulate repair or protective mechanisms.

[Key words: calcium oscillations, oligodendroglia, astrocyte, demyelinating disease, complement, injury, melittin, thapsigargin]

Oligodendroglia (ODG) are responsible for myelinating axons in the CNS. This role is crucial for the maintenance of saltatory conduction of nerve impulses, and agents that damage ODG

inevitably and profoundly disturb the function of myelinated axons. ODG and their myelin sheaths are selectively targeted during human and experimental inflammatory disease (Prineas et al., 1989). Although the immunological mechanisms of demyelination have yet to be fully elucidated, the inflammatory process culminates in loss of ODG and the phagocytosis of myelin lamellae by infiltrating or brain-derived macrophages (microglia). Based on observations made *in vitro* using purified ODG and cocultures of ODG and microglia, a role for serum complement in the pathogenesis of demyelination has recently been proposed (Scolding et al., 1989a, 1990; Compston et al., 1991; Zajicek et al., 1992a). Rat ODG fix and activate complement by the classical pathway in the absence of antibody (Scolding et al., 1989a; Wren and Noble, 1989). Most nucleated cells possess membrane-associated regulatory proteins that protect against autologous complement activation (reviewed by Lachmann, 1991). However, rat ODG are unique among glial cell types in that they lack adequate amounts of the complement regulatory protein CD59 (Wing et al., 1992). Complement activation usually leads to formation of the membrane attack complex (MAC) and cell lysis but most nucleated cells, including rat ODG, are resistant and exhibit a limited capacity for recovery. Osmotic protection against MAC attack is facilitated by rapid membrane vesicular repair and the voiding or inactivation of intercalated MAC, following which the cell remains viable (Scolding et al., 1989b). If these *in vitro* observations in the rat are representative of the intact human CNS, complement attack could provide a mechanism for the rapid and reversible functional deficit that characterizes brain inflammation and precedes the development of structural damage to the oligodendrocyte-myelin unit (Youl et al., 1991).

Interactions between complement and the cell membrane, and their consequences, are dependent on an initial $[Ca^{2+}]_i$ rise promoted by extracellular influx (Morgan, 1989). Previous studies, using obelin-loaded populations of ODG, have demonstrated that these cells also undergo a transient elevation in $[Ca^{2+}]_i$, associated with vesicular removal of MAC and depletion of intracellular levels of ATP. Furthermore, the calmodulin antagonist W7 [*N*-(6-aminoheptyl)-5-chloro-1-naphthalenesulfonamide] prevents recovery in a concentration-dependent manner (Scolding et al., 1989b, 1992). Subsequent measurement of $[Ca^{2+}]_i$ following attack by T-cell-derived perforin, which has structural and functional homologies with the terminal complement component C9, has indicated the complexity of Ca^{2+} responses in single cells to membrane injury (Jones et al., 1991). Since these and other results (Schlaepfer, 1977; Smith and Hall, 1988; Stys

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et al., 1990) demonstrate a pivotal role for $[Ca^{2+}]_i$ during ODG injury and repair, we have further explored ODG response to complement using the greater resolution afforded by the single-cell system and the use of microfluorimetry.

Materials and Methods

Primary glial cultures

ODG cultures were obtained from 7 d rat neonatal optic nerves as described by Raff et al. (1983). Approximately 1×10^4 cells were plated per 19 mm polylysine-coated coverslip and cultured in a 10% CO_2 atmosphere for 4 d prior to use in a low-serum [0.5% heat-inactivated (HI) fetal calf serum (FCS)] medium described by Bottenstein and Sato (1979). Staining with polyclonal antibody against galactocerebroside (galC) (Chemicon International) revealed that by this stage over 95% of cells were mature galC+ ODG. Type 2 astrocytes were found in small numbers in low-serum-containing cultures and in some instances their differentiation from oligodendroglial-type 2 astrocyte progenitor (O-2A) cells was encouraged using 10% serum-containing medium. After 4 d in culture this cell type demonstrated a stellate morphology and stained positively for glial fibrillary acidic protein (GFAP) and positively for the ganglioside marker A2B5 (American Type Culture Collection). Type 1 astrocytes were obtained by mechanical dissociation of 10-d-old mixed brain cultures, grown from postnatal day 2 whole-brain homogenates from which ODG, O-2A, and microglial cell types had been removed by shaking, as described elsewhere (Zajicek et al., 1992b). Type 1 astrocytes were recognized by their flattened morphology and their ability to bind GFAP.

Solutions. In all cases the standard perfusion solution (Tyrode's) contained (in mM) 145 NaCl, 10 glucose, 10 HEPES, 2.5 KCl, 1.5 $CaCl_2$, and 1.2 $MgCl_2$ brought to pH 7.3 with NaOH. Where appropriate, Ca^{2+} and Mg^{2+} were omitted and 10 mM EGTA added to provide a Ca^{2+} / Mg^{2+} -free perfusion medium. The following components were dissolved in Tyrode's unless otherwise stated: caffeine (Sigma) (10 mM), ionomycin (Sigma) [2 mM stock in dimethyl sulfoxide (DMSO)], calcium ionophore A23187 (Sigma) (2 mM stock in DMSO), thapsigargin (Calbiochem) (2 mM stock in DMSO), melittin (Sigma) (1 mg/ml stock in DMSO), phospholipase A_2 (PLA $_2$) (Sigma) (100 U/ml).

Microfluorimetry

ODG or astrocytes on coverslips were loaded in medium with the acetoxymethyl ester form of indo-1 (2 μ M) (Grynkiewicz et al., 1985) or Cremophor (Sigma)/indo-1 (10 μ l Cremophor/100 μ l indo-1 stock) for 30 min at 37°C. Coverslips were then rinsed in fresh medium and placed in Leibowitz L-15 medium for a further 30 min at room temperature. Coverslips were then mounted in a perfusion chamber containing Tyrode's solution and indo-1 fluorescence was monitored from single cells using a circular diaphragm of 20 μ m diameter. Excitation was at 360 nm and emission at 405 and 480 nm was detected simultaneously by two photomultipliers. Following background subtraction $[Ca^{2+}]_i$ was estimated from the 405:480 ratio as described previously by Benham (1989). Experiments were performed at 37°C using a heated, continuous perfusion system (flow rate, 1 ml/min). The difference between the bath temperature (which was constantly monitored) and the glycerol at the objective-coverslip interface was less than 3°C. It was not practicable to conduct all experiments at rat brain temperature because of the large quantities of reagents required for continuous perfusion. Once it was established that the oscillatory mechanism was robust at room temperature, remaining experiments were carried out between 21°C and 25°C using static bathing solutions, with reagent applications of approximately $10 \times$ bath volume.

Serum and complement components

Whole sera. Normal rat serum (NRS) was obtained by cardiac puncture from ether anesthetized adult Sprague-Dawley rats. Whole blood was pooled, clotted for 2 hr at 37°C in glass containers, and then centrifuged for 30 min at $1000 \times g$. Serum was removed and stored at $-70^\circ C$ until required and the same pool used throughout. Normal human serum (NHS) was obtained by venous puncture from eight individuals, pooled, treated, stored, and used as above.

Purified components. Purified rat C1 and C1q were prepared by allowing the C1q to bind to an IgG sepharose column as described by Wing et al., 1993. Briefly, human IgG was covalently coupled to CNBr-

activated sepharose, and then rabbit anti-human IgG antiserum was allowed to bind to human IgG on the column. For C1 preparation, normal rat serum was diluted 1:2 with veronal-buffered saline (VBS), which comprised 0.1 M sodium chloride, 4.94 mM sodium barbitone, 4 mM sodium azide, 0.15 mM calcium chloride, and 1 mM magnesium chloride, pH 7.4, and applied to the column. The unbound protein was washed off with VBS, and the bound C1 eluted with VBS plus 1 M NaCl. The C1 was concentrated by Amicon filtration to 1 mg/ml. For C1q preparation, serum was diluted 1:2 with VBS and divalent cations chelated with 10 mM EDTA to dissociate C1 into C1q and C1r/s before application to the column. Unbound protein was washed from the column as above, and the C1q eluted with 1 M NaCl. C1q was further purified and concentrated, following dialysis against 50 mM HEPES, 60 mM NaCl, and 10 mM EDTA at pH 7.8, on a Mono S cation exchange column and eluted with an NaCl gradient (Stemmer and Loos, 1984). Both preparations were shown to be hemolytically active by their ability to restore the lytic activity of a C1q-depleted serum against antibody-coated sheep erythrocytes (Fig. 1).

Human C9 was obtained as a by-product of a C5b-6 preparation and kindly supplied by R. Oldroyd (Molecular Immunopathology Unit, MRC Centre, Cambridge, UK). Briefly, acute phase serum was activated overnight with yeast, and then the pH was adjusted to 5.4 and dialyzed against water. The precipitate was redissolved in PBS plus azide, dialyzed against 0.01 M PO_4 , 0.05 M NaCl, 25% glycerol pH 7.0, applied to a DE-Sephacel column, and eluted with NaCl. The coeluting C5b-6 and C9 were then separated by gel filtration on a G200 Sephadex column. It was possible to restore almost 70% of the lytic activity obtained using NHS against antibody-coated sheep erythrocytes by reconstituting C9-depleted serum with C9 (Fig. 2).

Serum depletion. Cobra venom factor (CVF)-depleted serum was prepared by administration of a single intraperitoneal injection (1 μ g/gm body weight in phosphate-buffered saline) into an adult rat and complement activity monitored by tail bleeding at 2 hr intervals. Rat serum depleted of hemolytic activity was obtained by cardiac puncture 8 hr after CVF treatment (see Fig. 1). CVF was a kind gift of R. Oldroyd and was essentially phospholipase free. Rat serum was also prepared from indomethacin-treated (three intraperitoneal injections of 15 μ g/gm body weight over 3 d) adult rats to investigate the effects of arachidonate metabolites produced in serum during cardiac puncture.

Normal human serum was depleted of C9 by passage over an anti-C9-sepharose column, in the presence of 10 mM EDTA, and the protein-containing fractions were Amicon filtered to the original volume of the serum. This reagent was 99% depleted of C9 when tested by ELISA (R. Wurzner, personal communication).

Hemolytic assay for complement activity

To measure classical pathway activity, a 10% suspension of washed sheep erythrocytes was incubated with a complement-fixing rat anti-sheep erythrocyte antibody for 30 min at 4°C in complement-fixing diluent plus 0.1% gelatin (CFD). CFD was obtained from Oxoid, Basingstoke, England, and consists of VBS pH 7.2, with optimal concentrations of Mg^{2+} and Ca^{2+} for complement activation. The antibody-coated sheep erythrocytes were washed, and then resuspended to a 10% suspension in CFD. The antibody-coated sheep erythrocytes were incubated with a source of complement for 30 min at 37°C, and hemolysis measured by the release of hemoglobin into the supernatant at optical density 412 nm. The alternative pathway was measured by the lysis of a 10% suspension of guinea pig erythrocytes in alternative pathway buffer, which consisted of VBS plus 7 mM $MgCl_2$, 10 mM EGTA pH 7.2. Lysis was measured by the release of hemoglobin as described above.

Quantification of cell lysis

Propidium iodide (PI) (100 μ M final concentration) was added to assess membrane permeability, following exposure to membrane-damaging agents. PI is normally excluded by the intact plasma membrane but becomes intensely fluorescent following intercalation with DNA (Parks et al., 1986). Up to 500 cells per coverslip were counted to assess percentage of PI uptake.

Results

$[Ca^{2+}]_i$ oscillations are produced in ODG by sublytic concentrations of serum and precede recovery

Pooled rat serum was titrated to establish the maximum sublytic concentration (1:30) at which less than 15% of ODG became

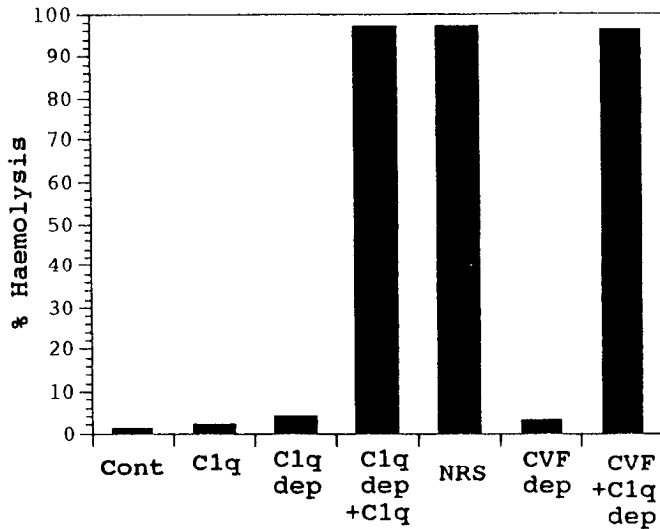


Figure 1. Incubation of antibody-coated sheep erythrocytes at 37°C with a 1:40 dilution of NRS resulted in 100% hemolysis. However, *in vivo* depletion with CVF yielded serum with only 6% of the hemolytic activity displayed by NRS. These cells were not lysed by purified C1q, and C1q-depleted serum resulted in only 8% hemolysis, suggesting low C1q contamination of this reagent. However, full hemolytic activity of the C1q-depleted serum could be achieved by the addition of C1q, confirming that it was only depleted of this component. Also full hemolytic activity was established when CVF-treated serum was supplemented with C1q-depleted serum, illustrating that the CVF-treated serum had been selectively depleted of C3 and C5-9.

PI positive following 60 min exposure (see Table 1). Serum was used at concentrations of between 1:5 and 1:200 in Tyrode's, direct from storage at -70°C . No qualitative differences in oscillatory patterns were noted in ODG exposed to serum between 21°C and 37°C; thus, experiments were conducted between 21°C and 25°C unless otherwise stated. In practice, the final resting $[\text{Ca}^{2+}]_i$ provided a good indicator of cell viability at the termination of each experiment, but in some cases cells used for microfluorimetry were assayed with PI immediately following recording, enabling comparison of final resting $[\text{Ca}^{2+}]_i$ with membrane permeability.

Resting $[\text{Ca}^{2+}]_i$ was recorded within a narrow range (75–125 nM) in all ODG studied. Exposure to NRS concentrations between 1:30 and 1:100 usually elicited an oscillatory $[\text{Ca}^{2+}]_i$ response with variable latency following addition of serum (between 30 sec and 11 min; see also Table 2). The majority of oscillatory responses (over 80%) elicited in ODG were complex with considerable variation in periodicity and peak amplitudes (between 500 nM and 3.0 μM) and oscillations invariably persisted for over 30 min before slowly returning to resting levels (Fig. 3). Two distinct patterns of oscillating response could be characterized. In one, oscillations appeared above an elevated basal $[\text{Ca}^{2+}]_i$ and only returned to resting levels once oscillations ceased ($n = 30$) (Fig. 3a); a second pattern of response was detected in less than 20% ($n = 6$) of ODG tested, where each oscillation returned, transiently, to the baseline before reinitiating (Fig. 3c). All ODG showing an oscillatory $[\text{Ca}^{2+}]_i$ response eventually returned close to resting levels in the continuous presence of 1:30 to 1:100 NRS ($n = 48$). Where oscillations were regular, the initial transient almost always peaked within 5 sec with a decay phase that was less rapid. The mean repeat between transients was 90 sec ($n = 12$) (Fig. 4a). Irregular oscillations were characterized by complex transients, usually

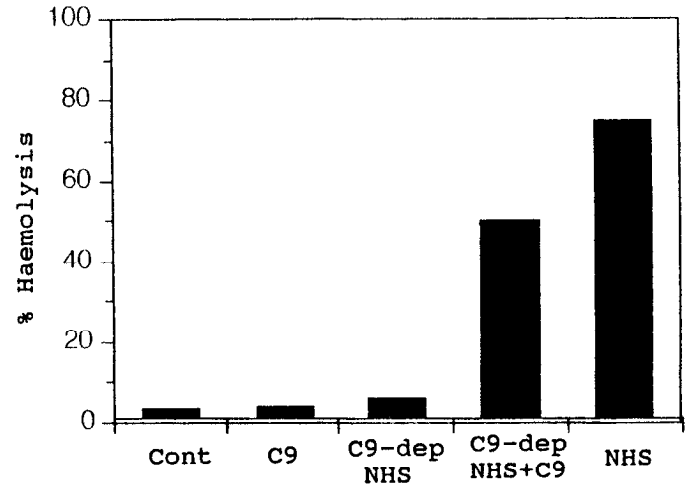


Figure 2. Residual hemolytic activity at 37°C following C9 depletion of human serum. Less than 5% of hemolytic activity obtainable using NHS remained following C9 depletion. Similarly, C9 alone caused almost no hemolysis of antibody-coated sheep erythrocytes. Reconstitution of C9-depleted serum with purified C9 resulted in better than 65% restitution of hemolysis.

reaching peak amplitude within 5 sec and having a less regular decay phase (Fig. 4b). Transient interperiods ranged between 30 and >100 sec.

A small number of ODG (see Fig. 3b, Table 2) underwent a monotonic rise in $[\text{Ca}^{2+}]_i$ leading to lysis within 30 min, following exposure to normally sublytic concentrations (1:30 to 1:60) of NRS ($n = 9$). In these cases, the initial increase in $[\text{Ca}^{2+}]_i$ was normally not as large as that seen preceding an oscillatory response. All ODG exposed to 1:5 NRS illustrated a similar response and became PI positive (data not shown). Nonoscillating cells were morphologically similar to oscillating ODG and initiated their response from a normal resting $[\text{Ca}^{2+}]_i$ level.

Concentrations of NHS between 1:30 and 1:50 were also able to provoke an oscillatory response in 67% ($n = 12$) of ODG (data not shown, but see Table 2). Mouse ODG isolated from whole brains also oscillated in response to 1:30 normal mouse serum or 1:30 NRS (data not shown; A. Wood and L. Ginsberg, unpublished observations).

Astrocytes respond to serum by transient elevations in $[\text{Ca}^{2+}]_i$, but their level of calcium excitability is temperature sensitive

Between 21°C and 25°C type 1 or type 2 astrocytes did not oscillate to 1:30 NRS. However, an immediate, consistent, and

Table 1.

Serum type	% PI uptake following 60 min exposure at 37°C
1:5 NRS fresh	97.0% ($n = 375$)
1:5 NRS freeze-thawed -70°C	92.3% ($n = 375$)
1:30 NRS fresh	42.7% ($n = 500$)
1:30 NRS* freeze-thawed -70°C	14.8% ($n = 500$)
1:30 NHS† freeze-thawed -70°C	12.6% ($n = 400$)

Data are percentage ODG lysis following treatment with NRS and NHS sera as assessed by permeability to PI. Percentage cell killing is reduced following a single freeze-thaw cycle from storage at -70°C . A single batch of pooled NHS† and NRS* was used throughout, directly from storage at -70°C .

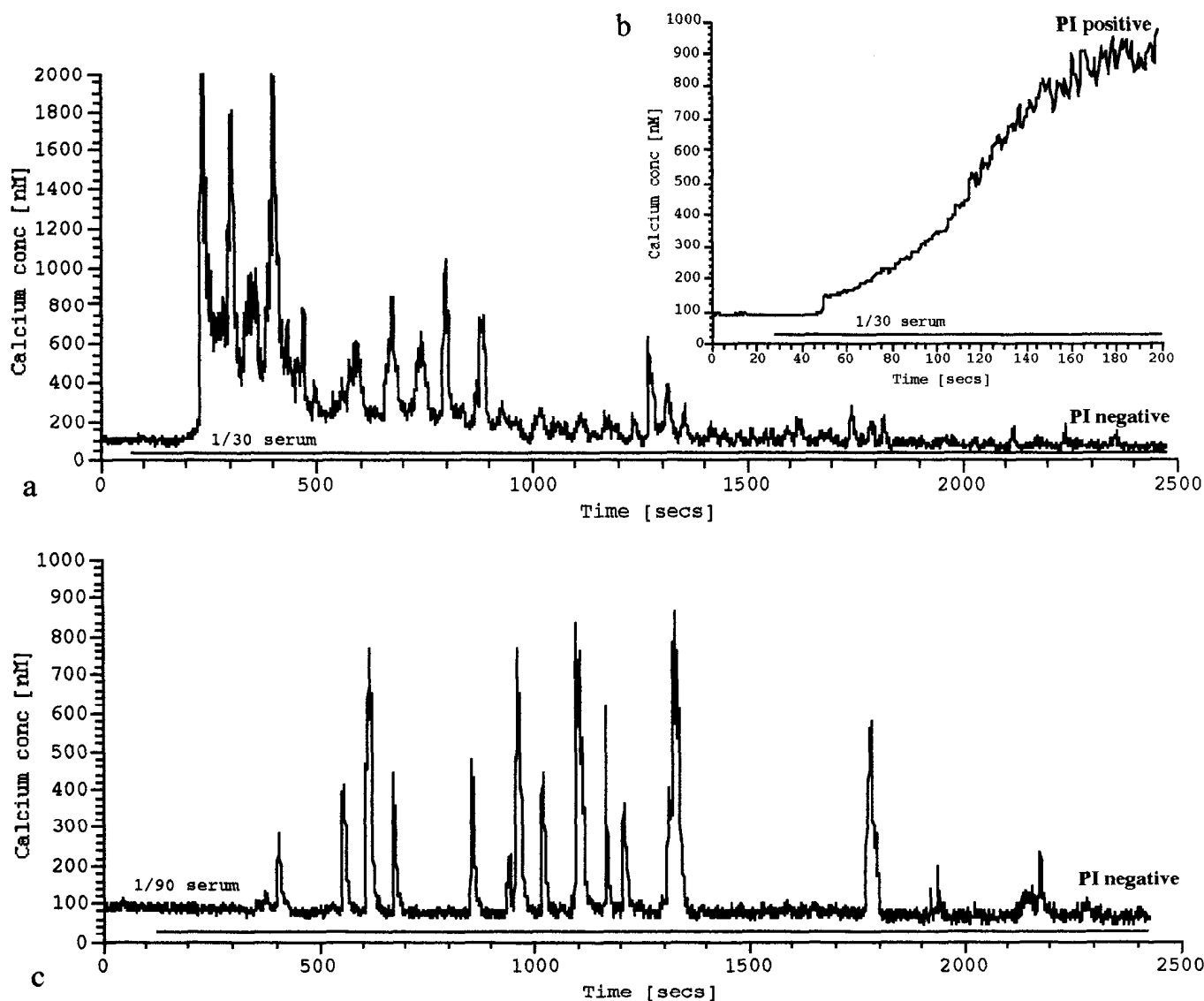


Figure 3. Serum-induced $[Ca^{2+}]_i$ oscillations show variable latencies and patterns following exposure to sublytic concentrations of NRS at 37°C. The ODG shown in *a* illustrated oscillations 2 min after addition of 1:30 serum, maintained an elevated basal $[Ca^{2+}]_i$ throughout, and was PI negative 45 min and 2 hr following recording ($n = 9$). The ODG illustrated in *b* did not oscillate and became PI positive only 5 min after the addition of 1:30 serum ($n = 2$). The latency before response to 1:90 serum, shown by the ODG in *c*, was greater (5 min) than for the cell depicted in *a*, but this ODG maintained a stable resting $[Ca^{2+}]_i$ during the periods between transients ($n = 2$).

large elevation in $[Ca^{2+}]_i$ was produced in all cells tested on exposure to 1:30 NRS ($n = 19$). $[Ca^{2+}]_i$ approached 1.5 μM followed by a slow, monotonic decrease to resting levels during a subsequent 30–50 min period (Fig. 5*a*). This pattern of Ca^{2+} rise occurred in response to HI NRS ($n = 3$), and could not be prevented by CVF ($n = 3$), C9 ($n = 2$), or C1 ($n = 2$) depletion of serum. At 37°C an oscillatory response was provoked in type 1 astrocytes following an initial transient similar to that seen at lower temperatures (Fig. 5*b*) in response to 1:5 NRS ($n = 8$), 1:5 HI NRS ($n = 6$), 10% HI FCS ($n = 11$), but not 1:30 NRS ($n = 6$), where the response was similar to that shown in Figure 5*a*. Type 2 astrocytes did not show substantive oscillations at 37°C ($n = 7$), and their response was similar to that illustrated in Figure 5*a*; occasionally one or two small transients followed the initial, immediate high-amplitude response (data not shown).

ODG $[Ca^{2+}]_i$ oscillations are not a response to hormones or growth factors contained in serum

Heat inactivation of serum (56°C for 30 min), a protocol that inactivates complement, prevented $[Ca^{2+}]_i$ oscillations in almost all ODG ($n = 28$). Heat inactivation also destroyed the lytic capacity of complement (a 1:30 dilution caused only 4.5% of red blood cell lysis obtainable with non-heat-treated serum), although 14% ($n = 4$ of 28) of ODG did respond to heat-treated serum with an immediate, but highly transient rise in $[Ca^{2+}]_i$ (data not shown, but see Table 2). Preincubation of ODG for 1–24 hr in either 10% HI FCS ($n = 4$) or 0.5–10% HI NRS ($n = 6$) did not prevent NRS-induced $[Ca^{2+}]_i$ oscillations, demonstrating that $[Ca^{2+}]_i$ oscillations in ODG were not part of the homeostatic response that follows a period of deprivation from serum.

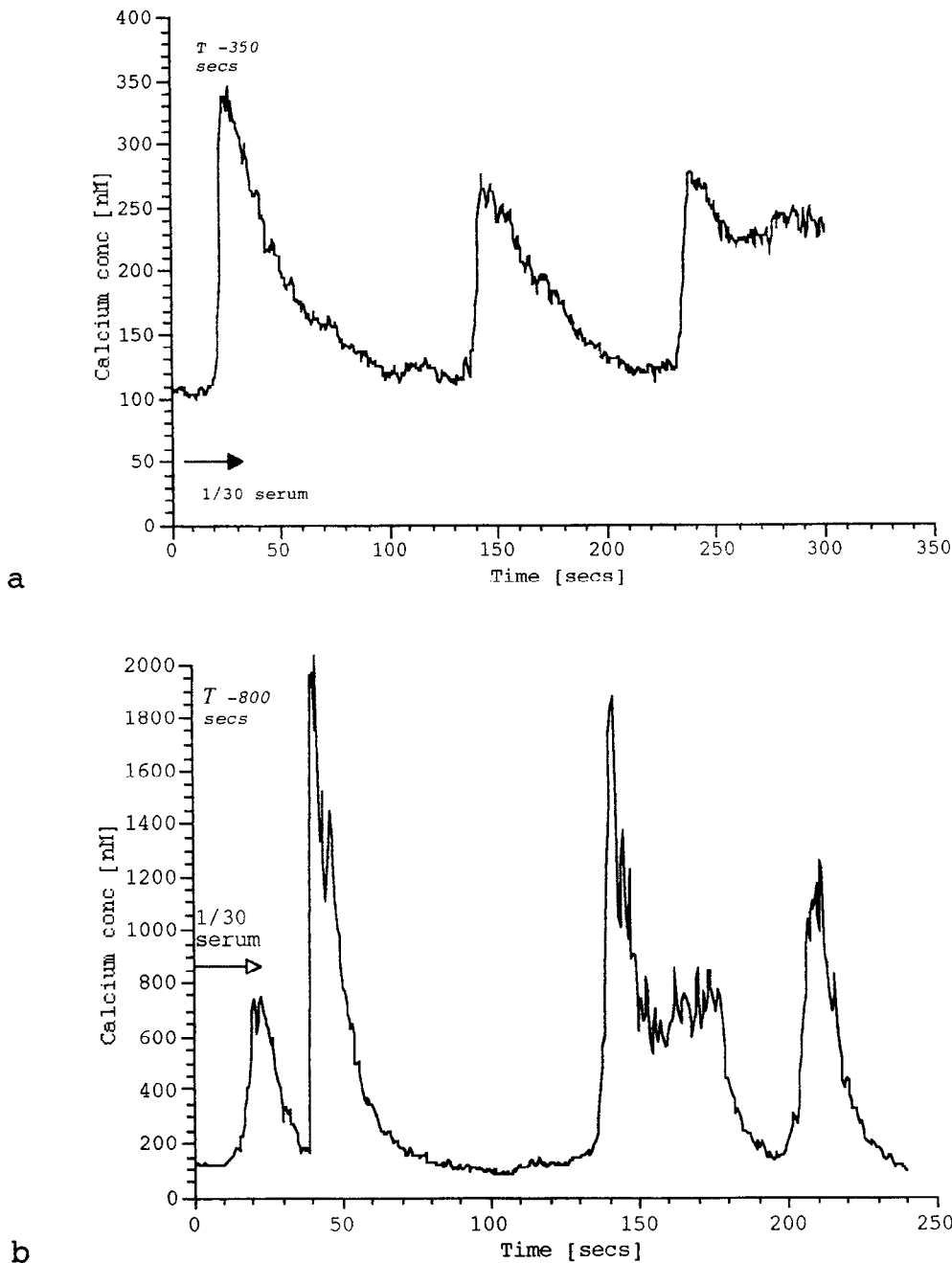


Figure 4. In a small number of cases the periodicity of $[Ca^{2+}]_i$ rise was regular (*a*). In these cells the amplitude of the transients was usually below 500 nM and oscillations subsided within 4–10 repeats. The decay phase was monotonic and could be described by a single exponential. However, the most common pattern of oscillatory $[Ca^{2+}]_i$ response is shown in *b*, where oscillations are irregular, of high amplitude and demonstrate variable decay patterns. (*T* = the period of exposure to serum previous to that shown on the figure).

$[Ca^{2+}]_i$ oscillations are not produced in response to arachidonic acid or its metabolites

Significant levels of arachidonic acid (AA) and its metabolites may be released during cardiac puncture. In order to exclude the possibility that these inflammatory mediators were causing $[Ca^{2+}]_i$ elevations, ODG were exposed both to AA and to serum derived from rats treated with indomethacin, a cyclo-oxygenase and lipoxygenase inhibitor (Shimizu and Wolfe, 1990). Removal of AA metabolites from serum did not prevent a normal oscillatory response ($n = 6$) (Fig. 6*a*). In contrast, AA alone (40 μ M) induced a transient but small elevation in $[Ca^{2+}]_i$ that was not seen using myristic acid (40 μ M) (Fig. 6*b*). Pretreatment with AA did not prevent an oscillatory response in ODG to 1:30 serum ($n = 3$).

Pre-opsonization by anti-galC causes ODG lysis in response to normally sublytic serum concentrations and C1q depletion prevents $[Ca^{2+}]_i$ oscillations

Preincubation of ODG at 37°C with a 70 μ g/ml concentration of anti-galC for 30 min prior to NRS exposure caused a single, large, and sustained elevation in $[Ca^{2+}]_i$ following addition of serum at concentrations between 1:60 and 1:100 ($n = 10$). Serum concentrations greater than 1:50 caused almost immediate lysis ($n = 6$). C1q-depleted serum did not provoke an oscillatory $[Ca^{2+}]_i$ response ($n = 13$) from opsonized or non-opsonized ODG (except in 13% of cases, where a small amplitude oscillatory response was detected, possibly caused by residual C1q activity; see Table 2, Fig. 1), suggesting that the serum-induced oscillations are produced via C1 activation of the complement

Table 2.

Serum type	Lyses erythrocytes		Effect on intracellular calcium			
	Alternative: guinea pig	Classical: sheep	No effect	Mono- tonic	Oscil- latory	Mean latency
1:5 NRS	Yes	Yes	0%	100%	0%	— (<i>n</i> = 8)
1:30 NRS	Yes	Yes	0%	11%	89%	4.2 min (<i>n</i> = 28)
1:30 HI NRS	No	No	86%	0%	14%	<1 min (<i>n</i> = 28)
1:30 NHS	Not tested	Yes	22%	0%	78%	5.7 min (<i>n</i> = 12)
1:30 Clq-depleted NRS	Yes	No	87%	0%	13%	4.0 min (<i>n</i> = 15)
1:30 CVF-depleted NRS	No	No	100%	0%	0%	— (<i>n</i> = 5)
1:30 C9-depleted NHS	Not tested	Yes	100%	0%	0%	— (<i>n</i> = 9)

A comparison of the hemolytic activity against antibody-coated sheep (classical pathway activation) and guinea pig (alternative pathway activation) red blood cells and a summary of the effect of the various sera used in this study on intracellular calcium. NHS, normal human serum; NRS, normal rat serum; HI NRS, heat inactivated normal rat serum.

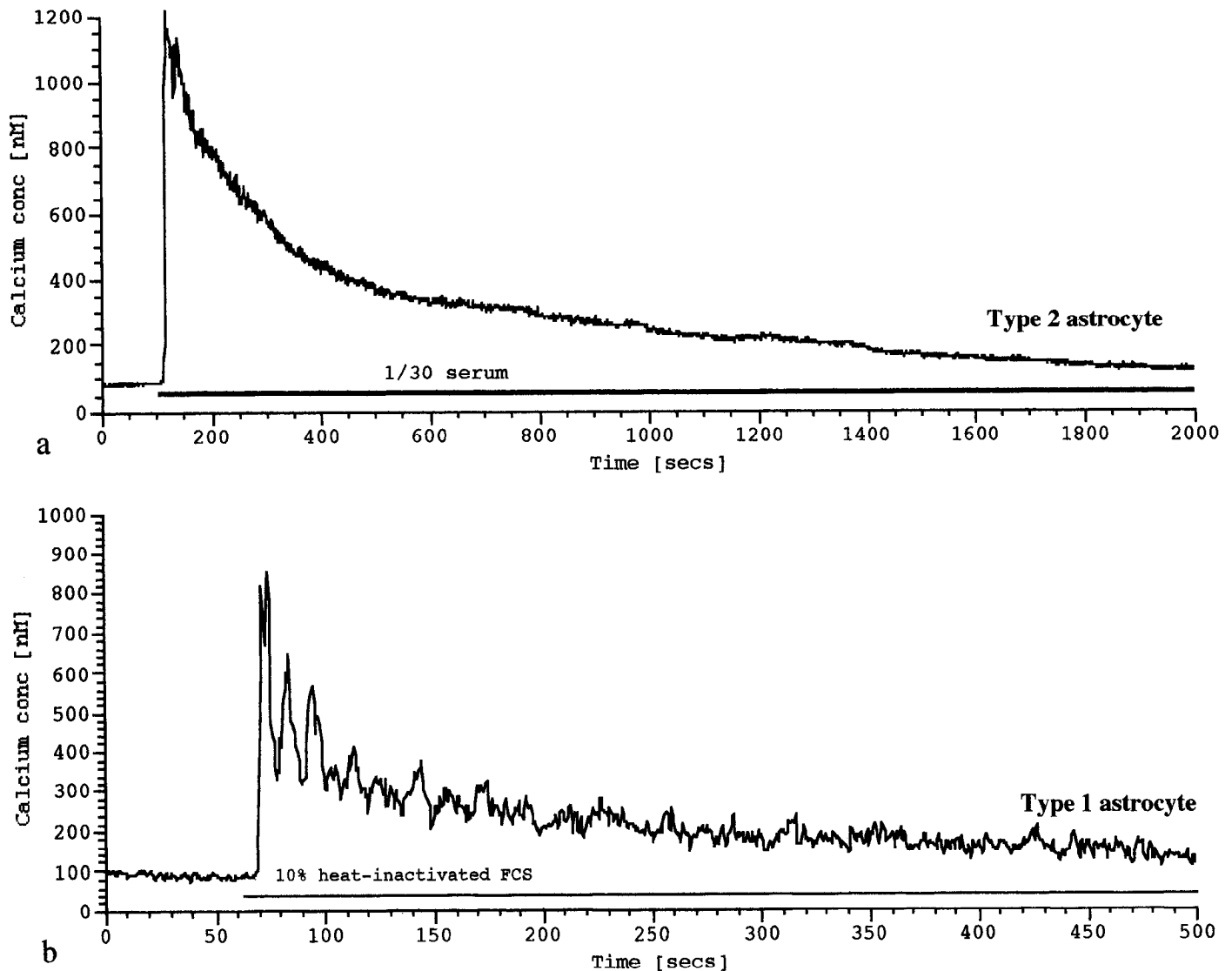


Figure 5. Between 21°C and 25°C the astrocyte response to serum concentrations between 1:5 and 1:30 was always characterized by an immediate and high-amplitude transient, followed by a slow monotonic decay phase, eventually reestablishing resting $[Ca^{2+}]_i$ levels within 50 min (*a*). At 37°C type 1 astrocytes showed increased Ca^{2+} excitability following exposure to 1:5 NRS, HI NRS, and 10% FCS (*b*). Prior to serum exposure resting $[Ca^{2+}]_i$ was always between 80 and 125 nM.

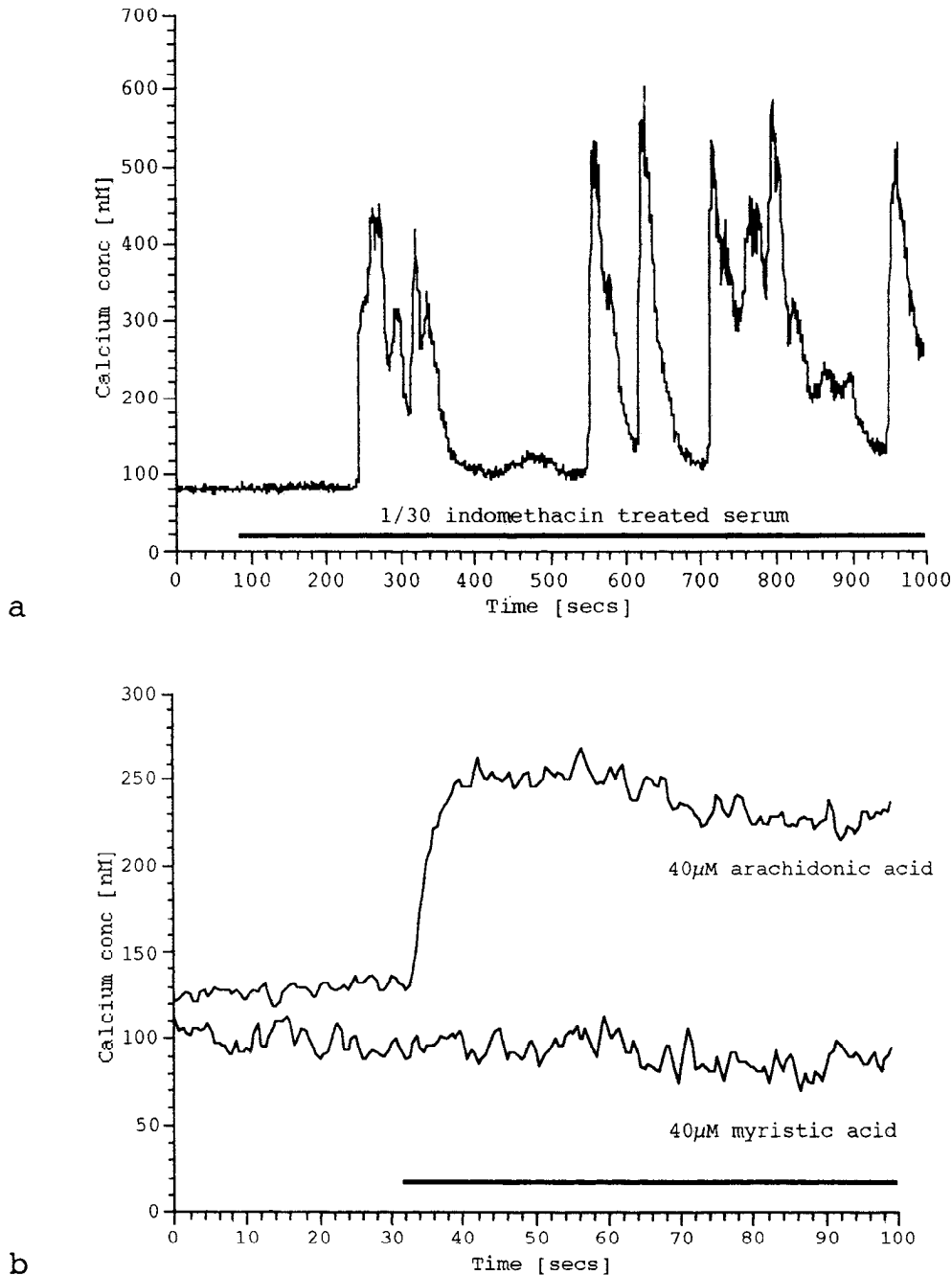


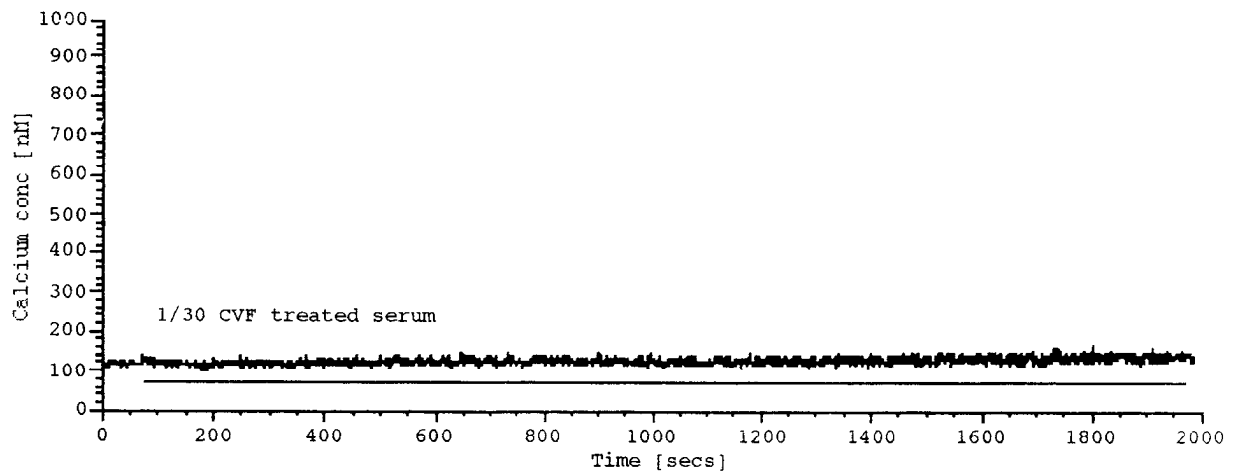
Figure 6. Indomethacin treatment of adult rats was unable to remove the oscillatory stimulus from serum derived by cardiac puncture (*a*). Following exposure to indomethacin-treated serum, ODG were provoked to oscillate with usual latency and pattern. However, AA (40 μ M) itself was able to provoke a modest and protracted rise in $[Ca^{2+}]_i$ that was not duplicated by myristic acid—a nonmetabolizable analog (*b*). The two traces illustrated in *b* are derived from different cells.

cascade. Exposure to purified C1 (up to 250 μ g/ml) ($n = 4$) or C1q (up to 185 μ g/ml) ($n = 6$) did not provoke a change in $[Ca^{2+}]_i$ (data not shown).

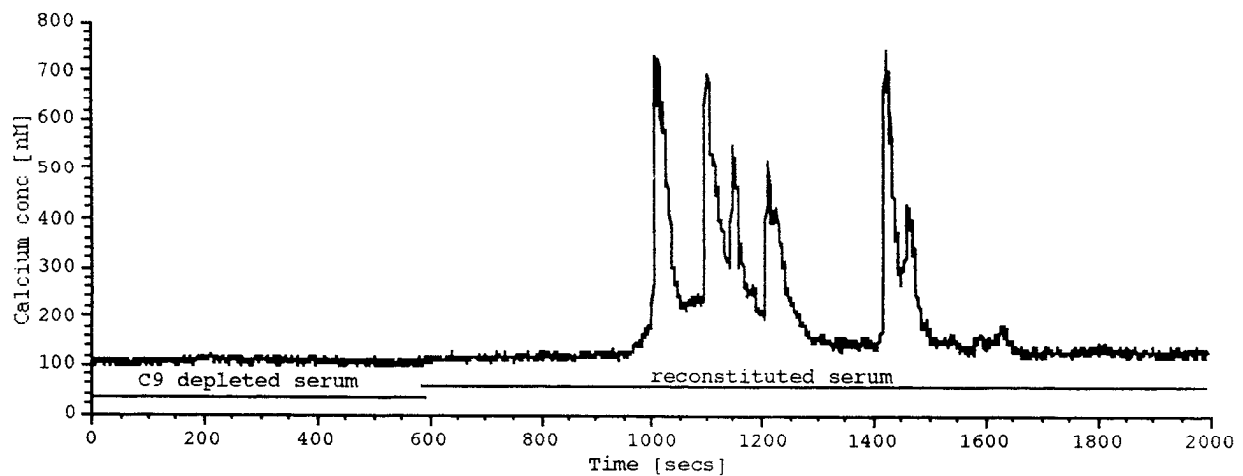
Oscillations are C9 dependent and can be reproduced by the C9-homologous polypeptide melittin

Human serum depleted of C9 was unable to elicit an oscillatory response ($n = 12$), even at concentrations up to 1:5 (data not shown, but see Table 2). Similarly, rat serum depleted of complement by CVF ($n = 5$) (Fig. 7*a*, Table 2) or C6 ($n = 2$), and C7 ($n = 3$) congenitally deficient human serum (generously provided by A. Orren and R. Wurzner, Molecular Immunopathology Unit, MRC Centre, Cambridge, UK; data not shown) failed to provoke oscillations. C9-depleted human serum re-

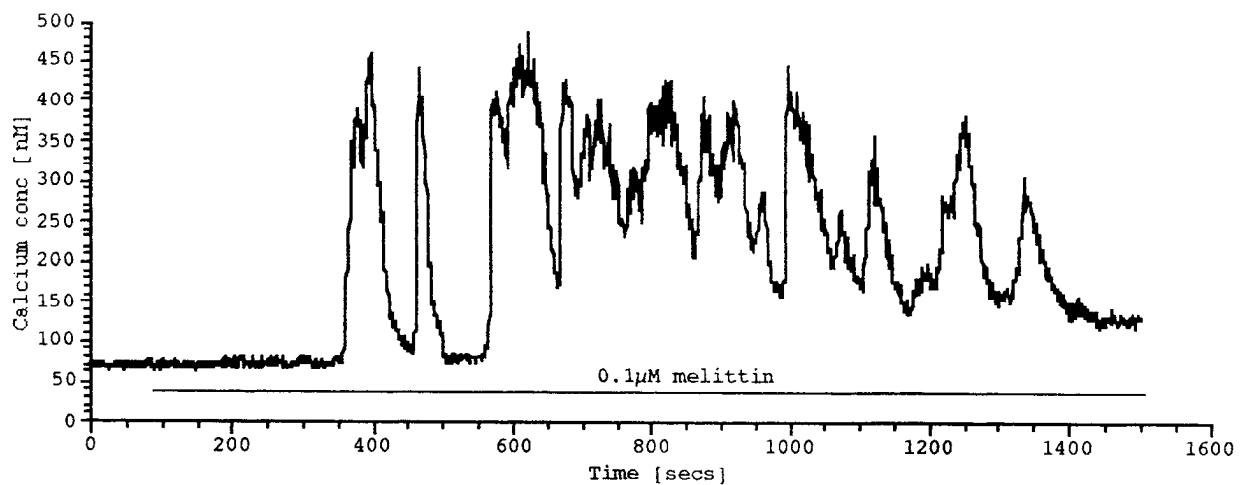
constituted with purified C9 (75 μ g/ml) was able to initiate an oscillatory response, illustrating that oscillations are MAC dependent and rely on the presence of the terminal complement component C9 ($n = 3$) (Fig. 7*b*). Furthermore, melittin (a polypeptide isolated from bee venom and sharing homology with C9) provoked an almost identical response ($n = 5$) (Fig. 7*c*). Commercially available melittin contains phospholipase A_2 (PLA $_2$) impurity; thus, PLA $_2$ (6–50 U/ml) and purified melittin (provided by J. Deighton, Molecular Immunopathology Unit, MRC Centre, Cambridge, UK) were used separately. PLA $_2$, up to 50 μ l/ml, was unable to stimulate oscillations ($n = 3$) and at higher concentrations produced monotonic increases in $[Ca^{2+}]_i$ leading to cell lysis ($n = 2$). PLA $_2$ -free melittin duplicated the response produced using commercially obtained melittin ($n = 4$) (data not shown).



a



b



c

Figure 7. CVF treatment of adult rats rapidly and completely removed the oscillatory stimulus from serum derived by cardiac puncture (a), even at concentrations up to 1:10. C9-depleted human serum was also unable to provoke an oscillatory $[Ca^{2+}]_i$ rise at concentrations up to 1:10. Reconstitution of serum by addition of purified human C9 *in vitro* provoked an oscillatory pattern of $[Ca^{2+}]_i$ rise following the usual period of delay (b). A complex oscillatory pattern of $[Ca^{2+}]_i$ rise could also be provoked by the C9-homologous polypeptide melittin (0.1 μM). Oscillations proceeded following latencies of between 4 and 6 min and usually returned to resting levels within 30 min (c). Higher concentrations of melittin provoked a monotonic $[Ca^{2+}]_i$ rise leading to lysis.

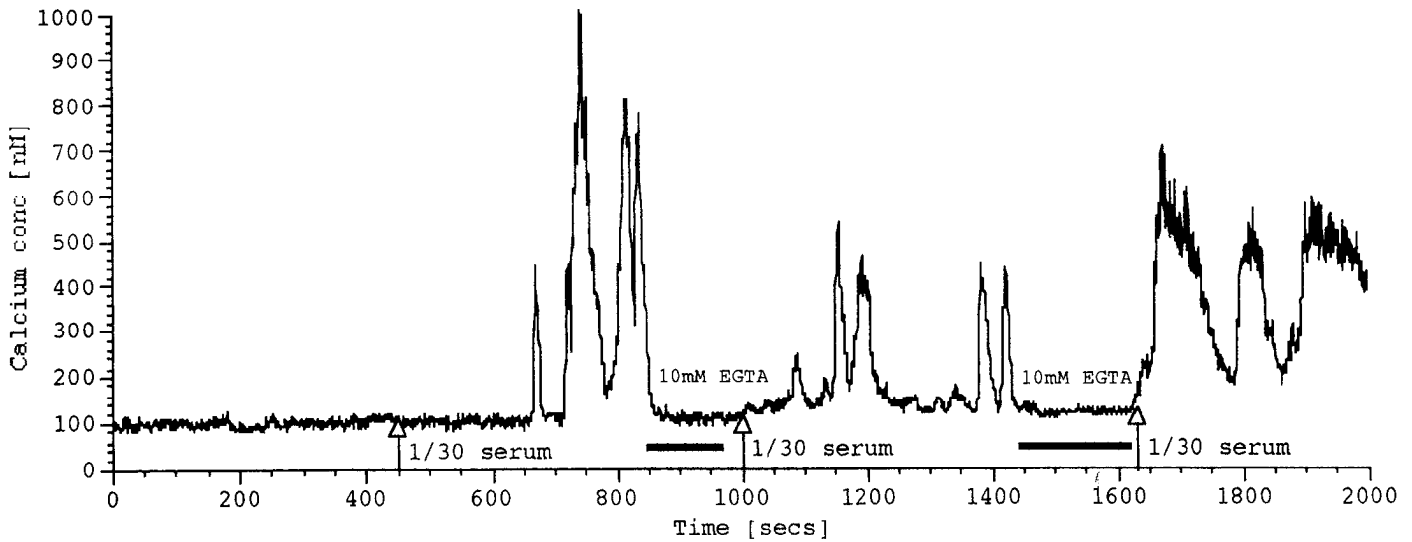


Figure 8. Removal of $[Ca^{2+}]_o$ from 1:30-diluted serum with either 10 mM EGTA or EDTA, following serum-induced $[Ca^{2+}]_i$ oscillations, immediately restored $[Ca^{2+}]_i$ to resting levels and prevented further transient increases. After washing off the divalent chelator with 1:30 serum in normal Tyrode's, an oscillatory $[Ca^{2+}]_i$ rise was immediately reinitiated.

Oscillations are prevented in the absence of $[Ca^{2+}]_o$ but cannot be reproduced by mechanically or ionophore-induced Ca^{2+} influx

Treatment of ODG with NRS, diluted in EGTA containing Tyrode's, failed to provoke an oscillatory response. However, initial cell surface activation of complement depends on the presence of divalent cations; therefore, in subsequent experiments $[Ca^{2+}]_o$ was chelated only after oscillations had begun. Removal of $[Ca^{2+}]_o$ caused an immediate cessation of oscillatory behavior ($n = 4$) (Fig. 8). Due to the short duration of Ca^{2+} influx, it was not possible to interfere with this component of the response but subsequent transients were always prevented. In contrast, removal of serum from the perfusion bath in the presence of $[Ca^{2+}]_o$ had no immediate effect on $[Ca^{2+}]_i$ oscillations (data not shown). The ionophores A23187 (1 μ M) ($n = 6$) (not shown) and ionomycin (200 nM) ($n = 14$), or a Ca^{2+} leak produced by wounding the cell with a micropipette ($n = 2$) all produced a transient rise in $[Ca^{2+}]_i$ (300–1200 nM) followed by a slow recovery phase, but no $[Ca^{2+}]_i$ oscillations (Fig. 9a,b).

Calcium channel antagonists do not prevent serum-induced ODG $[Ca^{2+}]_i$ oscillations

Pre-, continuous, or acute treatment of ODG cultures with the dihydropyridine calcium channel antagonists nifedipine and nimodipine (up to 10 μ M, data not shown) or SK&F 96365 (up to 10 μ M), an antagonist of receptor-mediated Ca^{2+} entry (Merritt et al., 1990), did not prevent or interfere with normal serum-induced $[Ca^{2+}]_i$ oscillations ($n = 4$) (Fig. 10).

Thapsigargin prevents oscillations

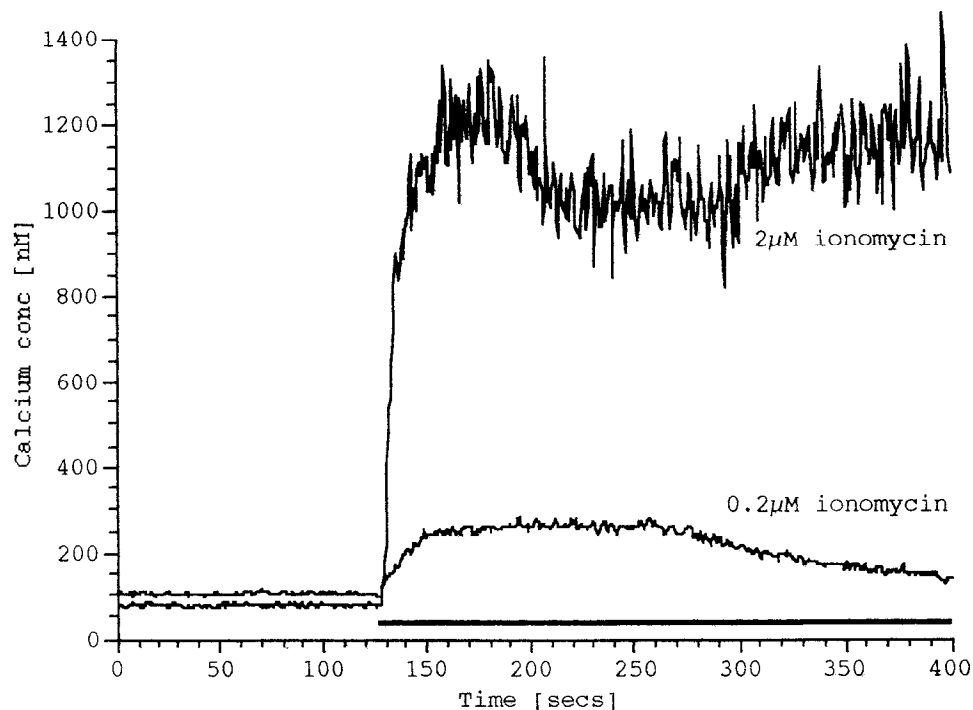
Caffeine (10 mM) produced a small (50–100 nM) rise in $[Ca^{2+}]_i$ in the presence of 10 mM EGTA, suggesting release from an intracellular store (data not shown). Pre- and continuous exposure to caffeine in the presence of divalent cations did not prevent or interfere with serum-induced $[Ca^{2+}]_i$ oscillations ($n = 3$) (data not shown). In contrast, the endoplasmic reticulum Ca^{2+} -ATPase inhibitor thapsigargin (Thastrup et al., 1990) in-

duced a large rise in $[Ca^{2+}]_i$, both in the presence and in the absence of $[Ca^{2+}]_o$ ($n = 5$) (Fig. 11a). In the presence of divalent cations and following pretreatment with thapsigargin, addition of 1:30 serum induced a protracted rise in $[Ca^{2+}]_i$ but no oscillations ($n = 3$) (Fig. 11b). The rise time of the initial complement-induced rise in $[Ca^{2+}]_i$ was considerably slowed in the presence of thapsigargin (Fig. 11c).

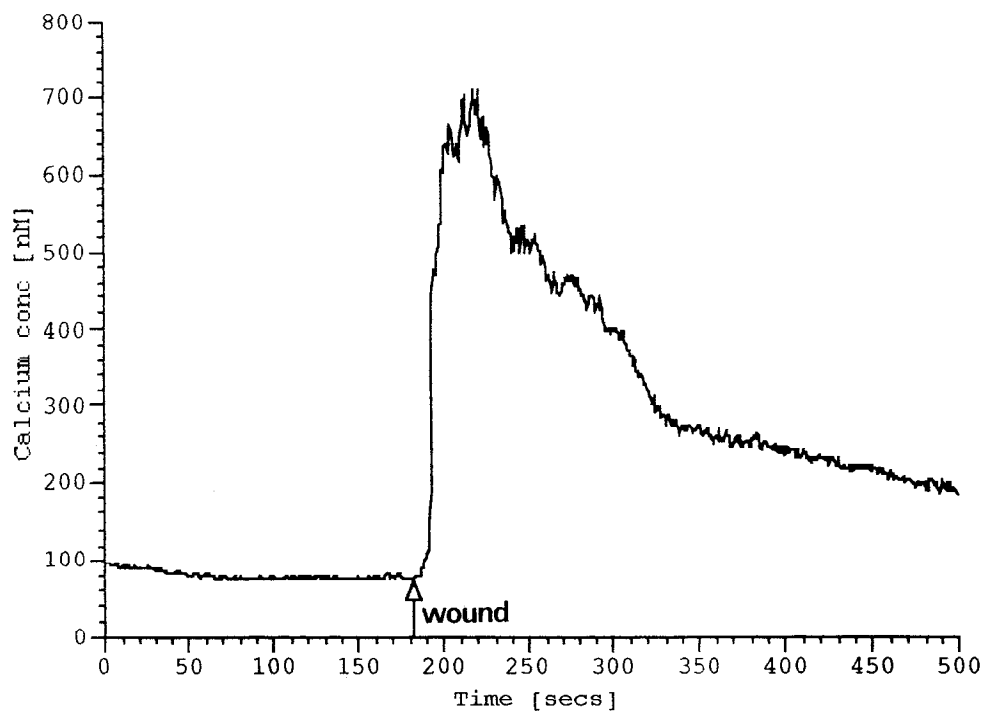
Discussion

This study demonstrates a complex and predictable oscillatory $[Ca^{2+}]_i$ response in ODG following exposure to serum-derived complement. Following sublethal injury, oscillations proceed for up to 45 min before a stable return to resting levels is established, after which the cell is temporarily refractory to further complement inducible changes in $[Ca^{2+}]_i$. Reversible injury by complement has previously been established in several cell types (reviewed by Morgan, 1989) and MAC-induced $[Ca^{2+}]_i$ oscillatory changes have been reported in Erlich ascites tumor cells using digital imaging fluorescence microscopy (Carney et al., 1990). In ODG, the response has previously been characterized as a transient elevation in $[Ca^{2+}]_i$, followed by vesicular repair of the plasma membrane and voiding of membrane damaging MAC (Scolding et al., 1989b). However, the complexity of the complement-induced change in $[Ca^{2+}]_i$ has not previously been fully appreciated, since population studies inevitably obscure individual transients if different cells are not oscillating synchronously. In this report we provide a detailed analysis of complement-induced $[Ca^{2+}]_i$ oscillations in ODG, a cell type that activates complement in the absence of antibody, and to our knowledge, the first reported observations of $[Ca^{2+}]_i$ oscillations to melittin.

There is considerable controversy surrounding the nature of the lesion produced by membrane insertion of MAC, and current descriptions of the morphological attributes of the MAC originate from different experimental approaches to the analysis of complement-mediated hemolysis (for conflicting hypotheses, see Bhakdi and Tranum-Jensen, 1991; Esser, 1991). The sim-



a



b

Figure 9. The calcium ionophore ionomycin was unable to induce $[Ca^{2+}]_i$ oscillations but provoked a concentration-dependent monotonic rise in $[Ca^{2+}]_i$ at concentrations between 100 nM and 2 μ M (*a*). Mechanically induced wounds made by transient stabbing with a micropipette were similarly unable to provoke $[Ca^{2+}]_i$ oscillations in ODG (*b*). Arrow indicates time of mechanical wounding.

ilarity in pattern of ODG $[Ca^{2+}]_i$ oscillations in response both to complement and to melittin suggests that each may exert a similar effect on the membrane. Melittin is a water-soluble peptide of 26 amino acids and is considered to form a lesion by distortion and aggregation of membrane lipids, creating a membrane permeabilization described as a "leaky patch." Comparison of melittin and human C9 amino acid sequences reveals

areas of complete homology, and anti-melittin antibodies are able to inhibit complement-mediated lysis of erythrocytes (Laine et al., 1988). Exposure of ODG to purified C9 alone does not affect $[Ca^{2+}]_i$, suggesting that oscillations may be a unique response to a leaky patch-type lesion.

The oscillations produced in ODG by melittin and complement are critically (but not solely; see below) dependent on Ca^{2+}

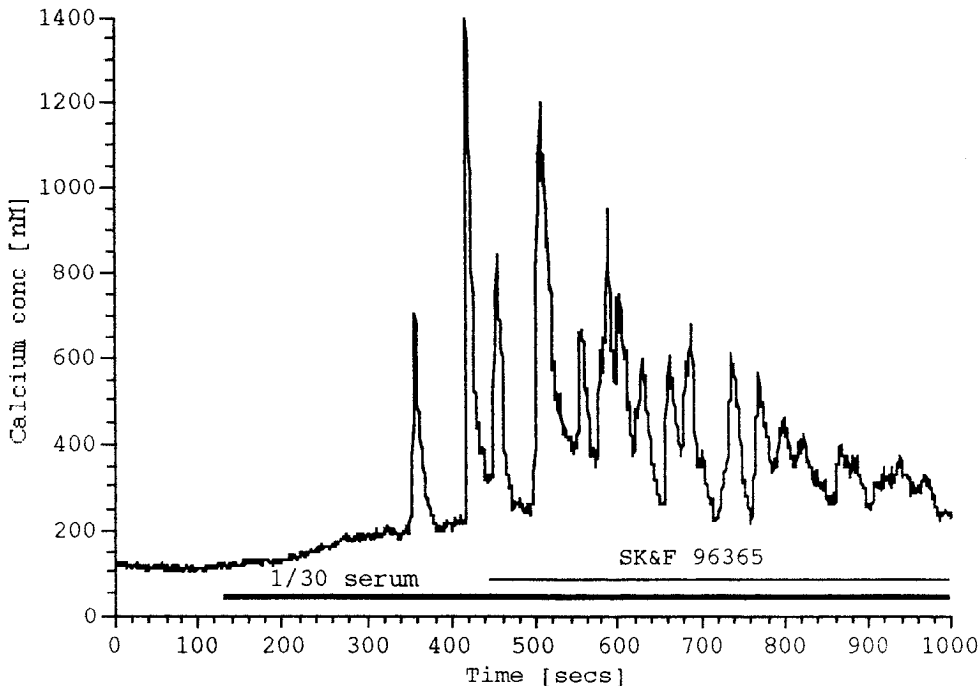


Figure 10. The receptor-mediated Ca^{2+} entry antagonist SK&F 96365 was unable to inhibit serum-induced $[\text{Ca}^{2+}]_i$ oscillations at concentrations up to $10 \mu\text{M}$.

influx across the plasma membrane, since removal of $[\text{Ca}^{2+}]_o$ immediately abolished oscillations. Cyclical Ca^{2+} entry is unlikely to involve activation of voltage-sensitive Ca^{2+} channels, since oscillations are unaffected by dihydropyridine analogs. However, periodic Ca^{2+} influx could be generated by functional instability of the MAC itself. Patch-clamp analysis of individual complement-induced channels in antibody-coated skeletal muscle cells has shown that "pores" are highly transitory, undergoing rapid structural transitions between conducting and nonconducting states (Jackson et al., 1981). Individual pore conductances up to 90 pS have been recorded and are calculated to provide a potential rate of intracellular ion increase of $100 \mu\text{M}/\text{sec}$ at a membrane potential of -10 mV . Using this example, it would only require a small number of pores to open synchronously to elevate $[\text{Ca}^{2+}]_i$ by $1\text{--}2 \mu\text{M}$ within a few seconds—the level and time course displayed by ODG oscillations. Duration and synchronicity of channel opening might therefore create the cyclical rise in $[\text{Ca}^{2+}]_i$. The frequency of opening would in turn determine whether each transient elevation is restored to baseline before reactivation, since rapidly forming channels would overwhelm pumping and sequestration mechanisms, and create oscillations similar to those illustrated in Figure 3a. Attempts to address whether oscillatory changes in membrane conductance accompanied $[\text{Ca}^{2+}]_i$ rises were unsuccessful, either in whole-cell patch or amphotericin-permeabilized patch modes, because we were unable to maintain stable patch-clamp recordings for sufficient duration to complete the experiments.

In this study we did not attempt to demonstrate complement-induced release of $[\text{Ca}^{2+}]_i$ from intracellular stores in the absence of $[\text{Ca}^{2+}]_o$ because initiation of the complement cascade requires the presence of Ca^{2+} . Previously, a complement-induced rise in $[\text{Ca}^{2+}]_i$ has been demonstrated in obelin-loaded populations of ODG in the presence of 0.5 mM EGTA (Scolding et al., 1990) using a "reactive lysis" system (the complement cascade is initiated in the presence of Ca^{2+} using C8- and C9-depleted serum; subsequently, these terminal components can be added to form

a functional MAC in the presence of EGTA); also, MAC-induced $[\text{Ca}^{2+}]_i$ release from intracellular stores has been shown in neutrophils (Morgan and Campbell, 1985). In both these studies, release from intracellular stores was modest by comparison with MAC-induced influx across the plasma membrane. However, both G-protein activation and diacylglycerol production have been demonstrated in a human lymphoblastoid cell line exposed to C5b-7, which demonstrates the initiation of phosphoinositide hydrolysis in the absence of a functional Ca^{2+} pore (Niculescu et al., 1992).

Intracellular stores must play a critical role during complement-induced $[\text{Ca}^{2+}]_i$ oscillations in ODG, as demonstrated by thapsigargin inhibition of the endoplasmic reticular Ca^{2+} -ATPase and the inability of either Ca^{2+} ionophores or mechanical wounding to initiate oscillations by a simple Ca^{2+} influx. Although caffeine treatment did not significantly alter resting $[\text{Ca}^{2+}]_i$, thapsigargin produced a transient peak and stable plateau in resting $[\text{Ca}^{2+}]_i$, and transformed complement-induced $[\text{Ca}^{2+}]_i$ oscillations into a sustained Ca^{2+} signal. The initial elevation in $[\text{Ca}^{2+}]_i$ provoked by complement following thapsigargin treatment is of the order of 500 nM , demonstrating significant $[\text{Ca}^{2+}]_i$ elevation during continuous depletion and thus independence of endoplasmic reticular stores. However, the rising phase was considerably slowed (Fig. 11c,) suggesting that Ca^{2+} -induced Ca^{2+} release from intracellular stores may be necessary to provoke oscillations. Furthermore, the sustained elevation of $[\text{Ca}^{2+}]_i$ in the presence of thapsigargin suggests that endoplasmic reticular stores may rapidly sequester Ca^{2+} in the intervals between transients.

Since the supply of complement components during oscillations should not have been a limiting factor, it is interesting to speculate on the cause of the gradual damping of transients and subsequent refractory phase against further MAC attack. In ODG, vesiculation is initiated within minutes of exposure to complement and is accompanied by a significant depletion in levels of ATP. The vesicular response is critically dependent on a rise in $[\text{Ca}^{2+}]_i$ and can be demonstrated in response to ionophores as

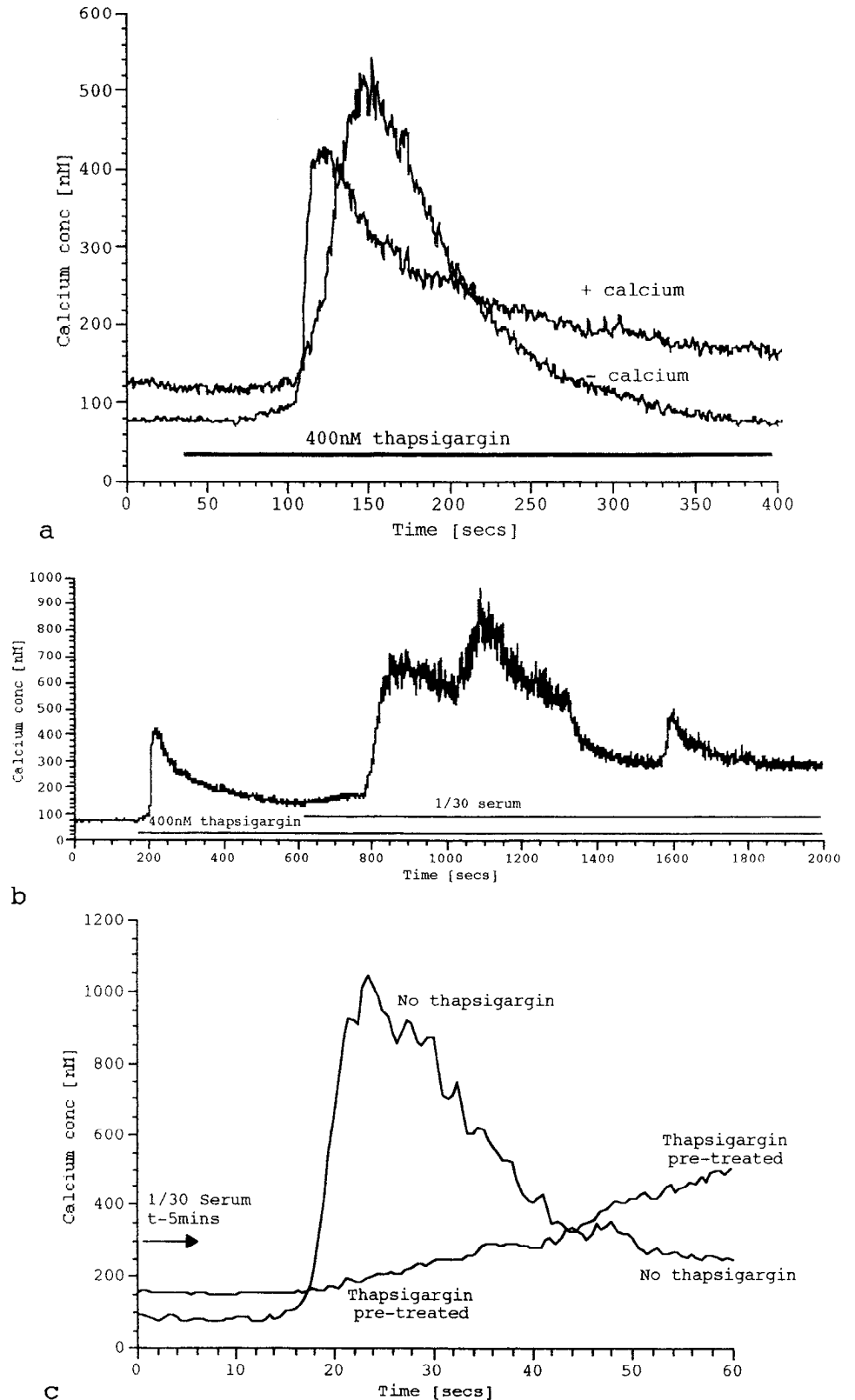


Figure 11. Concentrations of the endoplasmic reticular Ca^{2+} -ATPase inhibitor thapsigargin between 100 and 400 nM were able to precipitate a significant and concentration-dependent rise in $[\text{Ca}^{2+}]_i$, both in the absence and in the presence of $[\text{Ca}^{2+}]_o$. In the absence of $[\text{Ca}^{2+}]_o$, the thapsigargin-induced increase in $[\text{Ca}^{2+}]_i$ was less rapid but often pumped down marginally below resting $[\text{Ca}^{2+}]_i$ levels and more rapidly than in the presence of $[\text{Ca}^{2+}]_o$ (a). Pretreatment with 200–400 nM thapsigargin prevented $[\text{Ca}^{2+}]_i$ oscillations but contributed to an elevated $[\text{Ca}^{2+}]_i$ signal (b). Thapsigargin pretreatment also dramatically reduced the slope of the initial serum-induced $[\text{Ca}^{2+}]_i$ transient (c). t = the period of exposure to serum prior to that shown in the figure.

well as complement. In this respect, calmodulin inhibition lowers the threshold for complement lysis by blocking the vesiculation process (Scolding et al., 1989b). In the presence of saturating amounts of complement components provided by continuous perfusion, vesicular repair alone should not prevent

further membrane damage by MAC unless complement can no longer bind to the membrane, preventing further initiation of the cascade, or membrane lipids required for MAC insertion become saturated. Alternatively, vesicular repair may temporarily inhibit or outpace mechanisms leading to functional MAC

insertion under conditions of sublytic complement attack. Sublytic complement attack improves resistance of human erythroleukemic cells to complement lysis. These cells rapidly synthesize large "complement-induced proteins" (LCIPs) (Reiter and Fishelson, 1992; Reiter et al., 1992); although a direct role for LCIP in complement-induced protection has not been demonstrated, similarities in the kinetics of activation and induction of protection are intriguing. Similar complement-induced $[Ca^{2+}]_i$ -triggered novel gene expression in ODG could induce temporary resistance to further MAC attack.

Intracellular Ca^{2+} oscillations and propagating Ca^{2+} waves have been observed in response to the CNS neurotransmitter glutamate in cultures of rat type 1 but not type 2 astrocytes (Cornell-Bell et al., 1990; Jensen and Chiu, 1990, 1991) and within astrocytic networks from cultured slices of rat hippocampal tissue (Dani et al., 1992). Ca^{2+} oscillations have also been shown in astrocytes cultured from and in slices of rat supra-chiasmatic nucleus in response to cerebrospinal fluid, FCS, and fetal bovine serum (van den Pol et al., 1992). In our study the astroglial response to serum was immediate and characterized, at room temperatures, by a single large-amplitude transient. At 37°C type 1 but not type 2 astrocytes exhibited small-amplitude oscillations continuing for as long as 60 min following the initial high-amplitude transient. The increased Ca^{2+} excitability shown by type 1 astrocytes at 37°C suggests that a different mechanism may underlie the oscillatory behavior of this cell type compared with the temperature-insensitive oscillations exhibited by ODG to complement. Astrocyte responses to serum were not due to complement, since they could not be prevented by complement depletion of serum. Growth factor receptors are known to use $[Ca^{2+}]_i$ as a second messenger (Meldolesi et al., 1991) and it is possible that this type of response occurs after reexposure to serum following upregulation of growth factor receptors on the cell surface in low-serum-containing medium. Astrocytes are not susceptible to complement attack since they do not activate complement and possess complement regulatory proteins that interfere with the deposition of C3 (Gordon et al., 1992). However, these cells can be made susceptible to complement attack by phosphatidyl inositol-specific phospholipase C removal of glycoposphatidyl inositol-linked complement regulatory proteins from their surface (Wing et al., 1992a) and it would be interesting to investigate the effect of complement attack on $[Ca^{2+}]_i$ in unprotected astrocytes.

The CNS has traditionally been considered an immunologically privileged site; thus, exposure to complement was unlikely without prior damage to the blood-brain barrier. However, the presence of lymphatic-like capillaries in the brain may provide natural, untraumatized entry for lymphoid cells (Prineas, 1979) and low numbers of T-lymphocytes in brains with intact blood brain endothelia have been detected (Boos et al., 1983). Major histocompatibility complex antigens can be upregulated on astrocytes, ODG, and microglia *in vitro* and *in vivo* with γ -interferon (Wong et al., 1984). Complement proteins C1–C9 are present in normal human cerebrospinal fluid (Sano, 1985) and C3, C4, factor B, and factor H mRNA expression has been induced in cultures of astrocytes (Levi-Strauss and Mallat, 1987; Barnum et al., 1992; Rus et al., 1992). Taken together, these findings indicate a substantial degree of immune competence within the untraumatized CNS, which given the intrinsic sensitivity of ODG to complement may result in inadvertent opsonization of ODG surface membranes and injury of the ODG-myelin unit. Complement-induced ODG Ca^{2+} response is also

likely to have wider implications than only dictating individual recovery from Ca^{2+} overload, since complement-provoked release of inflammatory mediators from sublethally injured ODG may have profound consequences. A number of studies have demonstrated metabolism of AA and release of leukotrienes in response to sublytic C5b-9 and several of these compounds are metabolically active (Imagawa et al., 1983; Shirazi et al., 1986, 1989; Shimizu and Wolfe, 1990). Although evidence that AA metabolites function as intracellular messengers is still equivocal (but see Peppelenbosch et al., 1992), release of these compounds by injured ODG could affect neighboring neurons and glia. In this respect, AA was shown to provoke a small elevation in $[Ca^{2+}]_i$, both in this study and in ODG isolated from mice (Soliven et al., 1991); we are currently addressing the effects of metabolites of AA on $[Ca^{2+}]_i$ in ODG and myelinating cultures.

Our present results suggest that the consequences of complement assault on ODG are likely to depend on the precise nature of the ensuing Ca^{2+} overload. Sublethally injured ODG attained higher $[Ca^{2+}]_i$ levels than those ODG that were lysed in the presence of complement. This Ca^{2+} paradox can be rationalized if the $[Ca^{2+}]_i$ signal is able to stimulate repair mechanisms without disrupting Ca^{2+} homeostasis. Degradative enzymes are known to become activated during protracted elevations of Ca^{2+} and ODG possess myelin-specific Ca^{2+} -activated enzymes, including a basic protein kinase and a neutral protease responsible for myelin basic protein hydrolysis (Banik et al., 1985). Transient elevations of $[Ca^{2+}]_i$ into the micromolar range could avoid the activation of some enzymes while triggering the cytoskeletal/exocytotic machinery required for vesiculation and repair (Knight et al., 1989). Differential enzyme activation could be controlled by both the amplitude and frequency of these $[Ca^{2+}]_i$ transients (Rink and Jacob, 1989).

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